VOLUME 1

CURRENT PROTOCOLS IN MOLECULAR BIOLOGY

MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

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VOLUME 1

John Wiley & Sons, Inc.

CORE 14 (S41)

Library of Congress Cataloging in Publication Data:

Current protocols in molecular biology. 3 vols.

1. Molecular biology—Technique. 2. Molecular biology—Laboratory manuals. I. Ausubel, Frederick M.

QH506.C87 1987 574.8'8'028 87-21033

ISBN 0-471-50338-X

Printed in the United States of America

20 19 18 17 16 15 14

different amounts of hybrid[redacted]NA; if this occurs, the relative intensities displayed by two dots after hybridization will not be representative of the amount of target DNA that each contains.

The protocols for blotting uncharged nylon and nitrocellulose membranes attempt to ensure complete denaturation through the use of two denaturation steps—a heat denaturation before application to the membrane and an alkaline denaturation after application. Heat denaturation on its own is rarely adequate, as the DNA can renature fairly extensively before application to the membrane, even if plunged into ice on removal from the incubator. Blotting, whether manual or with a manifold, takes time, with some samples being blotted more quickly than others, so differential renaturation is a possibility. The second denaturation step, when the membrane is placed on a filter paper soaked in alkali, is intended to bring all the DNA back to an equal standing. Note that these problems do not arise with alkaline blotting onto positively charged nylon, as the high pH of the blotting solution maintains the DNA in a denatured state. Alkaline blotting is therefore the method of choice for DNA dot and slot blots where comparisons between different samples are to be made.

A second variable results from the purity of the DNA samples. With a Southern transfer, the gel electrophoresis step helps to fractionate away impurities, so the DNA that is transferred is relatively clean. Dot/slot blotting with bulk DNA lacks the benefit of a gel fractionation step, and the resulting co-blotted impurities can have unpredictable effects on hybridization, possibly reducing signal by blocking access to the hybridization sites, or increasing signal by trapping the probe. This must be taken into account if the signal intensity is to be used to estimate the absolute amount of target DNA, through comparison with a control dilution series. Copy number reconstruction by dot blot analysis is particularly suspect, as comparison between blots of cellular and plasmid DNA are reliable only if both types of DNA are scrupulously purified.

Troubleshooting

As with Southern blotting (*UNIT 2.9A*), most problems with dot and slot blots become apparent only after hybridization[redacted]. The warning signs detailed in the commentary to *UNIT 2.9A* also hold for dot/slot blotting; other problems are described in *UNIT 2.10* (see Table 2.10.4 for troubleshooting).

Anticipated Results

The procedures yield a clear white membrane carrying applied DNA in amounts up the carrying capacity of the matrix (Table 2.9.1, *UNIT 2.9A*). No data is generated until the membrane is subjected to autoradiography; anticipated results of autoradiography are discussed in *UNIT 2.10*.

Time Considerations

A manifold or manual blot can be set up and ready for sample application in as little as 15 min. After sample application, it takes about 3 hr to complete the protocol with a nitrocellulose membrane (most of this being the baking step), 60 min with an uncharged nylon membrane, and 30 min with a positively charged nylon membrane. The rate-determining step is sample application. Manifold application of clean samples (where no blockages occur) takes 5 min, but application by hand can take several hours if the sample volume is large and multiple additions have to made.

Literature Cited

Kafatos, F.C., Jones, C.W., and Efstratiadis, A. 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucl. Acids Res.* 7:1541-1552.

Key Reference

Dyson, N.J. 1991. Immobilization of nucleic acids and hybridization analysis. *In* Essential Molecular Biology: A Practical Approach, Vol. 2 (T.A.Brown, ed.) pp. 111-156. IRL Press at Oxford University Press, Oxford.

Describes dot and slot blotting in some detail.

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Hybridization Analysis of DNA Blots

The principle of hybridization analysis is that a single-stranded DNA or RNA molecule of defined sequence (the "probe") can base-pair to a second DNA or RNA molecule that contains a complementary sequence (the "target"), with the stability of the hybrid depending on the extent of base pairing that occurs. Experimentally, the analysis is usually carried out with a probe that has been labeled and target DNA that has been immobilized on a membrane support. Hybridization analysis is sensitive and permits detection of single-copy genes in complex genomes. The technique has widespread applications in molecular biology.

The first stage in a hybridization experiment is to immobilize the denatured nucleic acids on a suitable solid support. Methods for achieving this with gel-fractionated and bulk DNA are described in *UNITS 2.9A & 2.9B*. The labeled probe is then applied in a solution that promotes hybridization. After a suitable incubation, the membrane is washed so that any nonspecifically bound probe is removed, leaving only probe that is base-paired to the target DNA. By controlling the stringency of the washing conditions, decisions can be made about whether to target sequences that are 100% complementary to the probe, or allow some mismatching so that sequences with lower degrees of similarity are also detected. The latter approach (heterologous probing) is used to study related sequences in a single or more than one genome.

Hybridization analysis was originally carried out with long (100 to 1000 bp), radioactively labeled DNA probes. Other types of probe (RNA, oligonucleotide) have more recently been introduced, as have nonradioactive labeling and detection strategies. In addition, improvements in understanding of the factors that influence hybrid stability and hybridization rate have led to a proliferation of reagents and protocols for hybridization analysis. Finding one's way through the maze can be a daunting task, especially as protocols that work well with one probe-target combination may not work so well if either member of the partnership is changed. The approach taken here is to present as the basic protocol an unsophisticated procedure for hybridization analysis with a radiolabeled DNA probe. Despite its lack of embellishments, the protocol gives acceptable results with Southern and dot blots on nitrocellulose and nylon (uncharged and charged) membranes. The alternate protocol describes a similar method for probing DNA blots with a radiolabeled RNA probe. A support protocol for stripping blots to ready them for reprobing is also provided.

Relevant units elsewhere in the manual include the following: *UNITS 3.18 & 3.19* describe the preparation of nonradioactive probes and their use in hybridization analysis; *UNIT 4.9* covers hybridization analysis of immobilized RNA; *UNIT 6.3* describes hybridization analysis of recombinant clone libraries; and *UNIT 6.4* explains how to use labeled oligonucleotides as hybridization probes.

These hybridization protocols should not be read in isolation. The commentary describes various modifications that can be introduced, including changes to prehybridization, hybridization, and wash solution formulations, and alterations to incubation times and conditions, the latter including a discussion of the wash conditions compatible with different degrees of stringency. The intention is provide the reader with sufficient data to make well-informed decisions about how to modify the basic and alternate protocols for specific applications.

CAUTION: Investigators should wear gloves for all procedures involving radioactivity and should be careful not to contaminate themselves and their clothing. When working with ^{32}P, investigators should frequently check themselves and the working area for

radioactivity using a hand [redacted]onitor. Any radioactive contamination [redacted]be cleaned up using appropriate procedures. Radioactive waste should be placed in appropriately designated areas for disposal. Follow the guidelines provided by your local radiation safety adviser.

HYBRIDIZATION ANALYSIS OF A DNA BLOT WITH A RADIOLABELED DNA PROBE

BASIC PROTOCOL

This protocol is suitable for hybridization analysis of Southern transfers (*UNIT 2.9A*) and dot and slot blots (*UNIT 2.9B*) with a radioactively labeled DNA probe 100 to 1000 bp in length. The steps employ nylon membranes (uncharged or positively charged) but are suitable for nitrocellulose if modified as described in the annotations. The commentary describes how to tailor the protocol for individual requirements.

A hybridization experiment can be divided into three stages. First, the membrane is incubated in a prehybridization solution containing reagents that block nonspecific DNA binding sites on its surface, thereby reducing background hybridization. In this protocol, the blocking agents are Denhardt solution and denatured salmon sperm DNA; alternatives are described in the commentary. In the second stage, the prehybridization solution is replaced by fresh buffer containing the labeled probe, and an overnight incubation is carried out to allow the probe to bind to target sequences in the immobilized DNA. During this hybridization step, the probe pairs not only with target sites that have 100% complementarity with the probe, but also with related sequences. In the final stage of the experiment the membrane is washed with a series of solutions that gradually remove bound probe molecules until only highly matched hybrids remain.

Materials

DNA to be used as probe
Aqueous prehybridization/hybridization (APH) solution, room temperature and 68°C
2× SSC/0.1% (w/v) SDS
0.2× SSC/0.1% (w/v) SDS, room temperature and 42°C
0.1× SSC/0.1% (w/v) SDS, 68°C
2× and 6× SSC (*APPENDIX 2*)
Hybridization oven (e.g., Hybridiser HB-1, Techne) *or* 68°C water bath or incubator
Hybridization tube *or* sealable bag and heat sealer
Additional reagents and equipment for DNA labeling by nick translation or random oligonucleotide priming (*UNIT 3.5*), measuring the specific activity of labeled DNA and separating unincorporated nucleotides from labeled DNA (*UNIT 3.4*), and autoradiography (*APPENDIX 3*)

1. Label the probe DNA to a specific activity of $>1 \times 10^8$ dpm/μg by nick translation or random oligonucleotide priming. Measure the specific activity and remove unincorporated nucleotides.

   The probe should be a double-stranded DNA fragment, ideally 100 to 1000 bp in length. Usually the probe DNA is obtained as a cloned fragment (Chapter 1) which is purified from the vector by restriction digestion (UNIT 3.1) followed by recovery from an agarose gel (UNIT 2.6).

2. Wet the membrane carrying the immobilized DNA in 6× SSC.

   The membrane is blotted as described in UNIT 2.9A. Do not handle the membrane: use clean blunt-ended forceps.

3. Place the [redacted]ane, DNA-side-up, in a hybridization [redacted]nd add ~1 ml APH solution per 10 cm^2 of membrane.

Prehybridization and hybridization are usually carried out in glass tubes in a commercial hybridization oven. Alternatively, a heat-sealable polyethylene bag can be used. The membrane should be placed in the bag, all edges sealed using a heat sealer, and a corner cut off. The APH solution is then pipetted into the bag through the cut corner and resealed.

4. Place the tube in the hybridization oven and incubate 3 hr with rotation at 68°C.

If using a bag, shake slowly in a suitable incubator or water bath.

If using a nylon membrane, reduce the prehybridization period to 15 min, but warm the prehybridization/hybridization solution to 68°C before adding to the membrane.

5. Denature the probe DNA by heating for 10 min in a water bath or incubator at 100°C. Place in ice.

Step 5 should be done immediately before step 6, with a minimum delay between them.

6. Pour the APH solution from the hybridization tube and replace with an equal volume of prewarmed (68°C) APH solution. Add denatured probe and incubate with rotation overnight at 68°C.

The probe concentration in the hybridization solution should be 10 ng/ml if the specific activity is 10^8 dpm/μg, or 2 ng/ml if the specific activity is 1×10^9 dpm/μg. If using a bag, cut off a corner, pour out the prehybridization solution, add the hybridization solution plus probe, and reseal. It is very difficult to avoid contaminating the bag sealer with radioactivity; furthermore, the sealing element (the part that gets contaminated) is often difficult to clean. Hybridization bags are therefore not recommended.

7. Pour out the APH solution, using the appropriate disposal method for radioactive waste, and add an equal volume of 2× SSC/0.1% SDS. Incubate with rotation for 10 min at room temperature, changing the wash solution after 5 min.

CAUTION: *All wash solutions must be treated as radioactive waste and disposed of appropriately.*

To reduce background, it may be beneficial to increase the volume of the wash solutions by 100%. If using a bag, transfer the membrane to a plastic box for the washes.

8. Replace the wash solution with an equal volume of 0.2× SSC/0.1% SDS and incubate with rotation 10 min at room temperature, changing the wash solution after 5 min (this is a low-stringency wash; see commentary).

9. If desired, carry out two further washes as described in step 8 using prewarmed (42°C) 0.2× SSC/0.1% SDS for 15 min each at 42°C (moderate-stringency wash).

10. If desired, carry out two further washes using prewarmed (68°C) 0.1× SSC/0.1% SDS for 15 min each at 68°C (high-stringency wash).

11. Pour off the final wash solution, rinse the membrane in 2× SSC at room temperature, and blot excess liquid. Wrap in plastic wrap.

Do not allow the membrane to dry out if it is to be reprobed.

12. Set up an autoradiograph (*APPENDIX 3*).

HYBRIDIZATION ANALYSIS OF A DNA BLOT WITH A RADIOLABELED RNA PROBE

ALTERNATE PROTOCOL

Purified RNA polymerases from bacteriophages such as SP6, T3, and T7 (*UNIT 3.8*) are very efficient at synthesizing RNA in vitro from DNA sequences cloned downstream of the appropriate phage promoter (Little and Jackson, 1987). Several micrograms of RNA can be obtained from 1 µg of DNA template in a 10-min reaction. If a radiolabeled ribonucleotide is added to the reaction mixture, the polymerase synthesizes uniformly labeled RNA with specific activities up to and beyond 10^9 dpm/µg. The fact that RNA probes are single-stranded gives them certain advantages over DNA probes. RNA probes do not need to be denatured before being added to the hybridization solution, and remain fully available for hybridization during the course of the incubation. In contrast, the "potency" of a double-stranded DNA probe gradually declines as the complementary strands of the probe reanneal during the hybridization reaction, reducing the amount of probe available to hybridize to the target.

The hybridization protocol for an RNA probe is not greatly different from that with labeled DNA. Formamide is usually included in the prehybridization/hybridization solutions, because in the presence of formamide an RNA-DNA hybrid is more stable than the equivalent DNA-DNA hybrid. Carrying out the hybridization with formamide also permits the incubation to be at a lower temperature without loss of stringency. Single-strand-specific RNases are added to one or more of the wash solutions to remove nonhybridized probe molecules, considerably reducing background.

The protocol includes details of probe preparation (Mundy et al., 1991). The hybridization procedure is suitable for both nitrocellulose and nylon membranes, though backgrounds may be higher with nylon.

Additional Materials

TE buffer, pH 8.0 (*APPENDIX 2*)
Labeling buffer
Nucleotide mix (see reagents and solutions)
200 mM dithiothreitol (DTT), freshly prepared
20 U/µl human placental ribonuclease inhibitor
[α-^{32}P]UTP: 20 mCi/ml (800 Ci/mmol) or 10 mCi/ml (400 Ci/mmol)
SP6 or T7 RNA polymerase (*UNIT 3.8*)
RNase-free DNase I (*UNIT 3.12*)
0.25 M EDTA, pH 8.0 (*APPENDIX 2*)
Formamide prehybridization/hybridization (FPH) solution
2× SSC (*APPENDIX 2*) containing 25 µg/ml RNase A + 10 U/ml RNase T1 (*UNIT 3.13*)
Additional reagents and equipment for cloning and purifying plasmid DNA (Chapter 1), phenol extraction and ethanol precipitation (*UNIT 2.1*), restriction digestion of DNA (*UNIT 3.1*), measuring the specific activity of and separating unincorporated nucleotides from labeled RNA (*UNIT 3.4*), and autoradiography (*APPENDIX 3*)

Prepare the RNA probe

1. Clone into a suitable vector (Table 2.10.1) the DNA fragment that will be transcribed into the RNA probe.

   DNA must be of high purity, so use a method that includes a CsCl/ethidium bromide equilibrium centrifugation step (UNIT 1.7).

2. Linearize the DNA by restriction digestion immediately downstream of the cloned fragment.

Linearization introduces an endpoint for RNA synthesis. This ensures that enzymes and substrates are not wasted by transcribing downstream vector DNA, and also increases the specificity of the probe by excluding unwanted sequences.

3. Purify the DNA from the restriction enzyme by phenol extraction and ethanol precipitation. Resuspend in TE buffer, pH 8.0, at a concentration of 1 mg/ml.

4. Mix the following at room temperature:

   4 µl labeling buffer
   1.5 µl nucleotide mix
   1 µl 200 mM DTT
   1 µl (20 U) human placental ribonuclease inhibitor
   2 µg purified plasmid DNA from step 3
   100 to 200 µCi [α-^{32}P]UTP
   H_2O to a final volume of 20 µl.

   These are mixed at room temperature, as the spermidine in the labeling buffer may precipitate on ice.

5. Add 5 U SP6 or T7 RNA polymerase. Incubate for 1 hr at 40°C for SP6 or at 37°C for T7.

6. Add 2 U RNase-free DNase I and incubate at 10 min 37°C. Stop the reaction by adding 2 µl of 0.25 M EDTA, pH 8.0.

   DNase treatment degrades the template. This step may not be necessary for probes to be used in hybridization analysis, but is worth doing to be on the safe side.

7. Measure the specific activity of the RNA by acid precipitation and remove unincorporated nucleotides by the spin-column procedure.

   The specific activity should be at least 7×10^8 dpm/µg, preferably >10^9 dpm/µg.

   The labeled probe can be stored at –20°C for 2 days before use.

Carry out hybridization analysis

8. Carry out the prehybridization incubation as described in steps 2 to 4 of the basic protocol, but use FPH solution and incubate at 42°C.

9. Replace the FPH solution with an equal volume of fresh prewarmed solution. Add the labeled probe and incubate overnight with rotation at 42°C.

   The probe concentration in the hybridization solution should be 1 to 5 ng/ml. Hybridizations in formamide solutions are carried out at lower temperatures than aqueous hybridizations. However, if background hybridization is a problem, raise the incubation temperature to 65°C.

10. Wash the membrane as described in steps 7 to 8 of the basic protocol.

11. Replace the wash solution with an equal volume of 2× SSC containing 25 µg/ml RNase A + 10 U/ml RNase T1; incubate with rotation for 30 min at room temperature.

    The RNase wash decreases background hybridization.

12. Carry out moderate- and high-stringency washes as desired, rinse the membrane in 2× SSC, and set up autoradiograph as in steps 9 to 12 of the basic protocol.

Table 2.10.1 Selected Cloning Vectors Incorporating Promoters for Bacteriophage RNA Polymerases

Vector	Size (bp)	Markers[a]	Promoters
pBluescript	2950	amp, *lacZ'*	T3, T7
pGEM series	2746-3223	amp, *lacZ'*	SP6, T7
pGEMEX-1	4200	amp	SP6, T3, T7
pSELECT-1	3422	tet, *lacZ'*	SP6, T7
pSP18, 19, 64, 65	2999-3010	amp	SP6
pSP70, 71, 72, 73	2417-2464	amp	SP6, T7
pSPORT1	4109	amp, *lacZ'*	SP6, T7
pT3/T7 series	2700, 2950	amp, *lacZ'*	T3, T7
pWE15	8800	amp, neo	T3, T7
pWE16	8800	amp, dhfr	T3, T7

[a]Abbreviations: amp, ampicillin resistance; dhfr, dihydrofolate reductase; *lacZ'*, β-galactosidase α-peptide; neo, neomycin phosphotransferase (kanamycin resistance); tet, tetracycline resistance.

REMOVAL OF PROBES FROM HYBRIDIZED MEMBRANES

SUPPORT PROTOCOL

If the DNA has been immobilized on the membrane by UV crosslinking (for uncharged nylon membranes) or by alkaline transfer (for positively charged nylon), the matrix-target DNA interaction (which is covalent in nature) is much stronger than the target-probe interaction (which results from hydrogen bonding). It is therefore possible to remove (or "strip off") the hybridized probe DNA without removing the membrane-bound target DNA. Nylon membranes can therefore be reused several times—a dozen reprobings are routinely possible. Hybridized probe DNA can also be stripped from nitrocellulose membranes, but the weakness of the hydrophobic interactions that bind the target DNA to the matrix, plus the fragility of nitrocellulose, limits the lifetime of these membranes to three reprobings at most.

This protocol describes three methods for probe stripping, in order of increasing harshness. The treatment that is needed depends on how tightly the probe anneals to the target, which in turn depends on the GC content of the probe and the number of base pairs that are formed. To start with, use the least harsh treatment, monitoring the results by autoradiography of the stripped membrane. If a hybridization signal can still be seen, move up to a harsher method. To strip probe from a northern membrane (with immobilized RNA), use the mild treatment only. Do not add NaOH to RNA; it will be destroyed.

Additional Materials

Mild stripping solution
Moderate stripping solution
0.4 M NaOH
0.1% (w/v) SDS, 100°C

CAUTION: Although the stripping solutions may not become highly radioactive, they should still be disposed of as radioactive waste.

1a. *Mild treatment:* Wash the membrane in several hundred milliliters of mild stripping solution for 2 hr at 65°C.

1b. *Moderate treatment:* Wash the membrane in 0.4 M NaOH for 30 min at 45°C. Then rinse twice in several hundred milliliters of moderate stripping solution for 10 min at room temperature.

1c. *Harsh t[illegible]gt:* Pour several hundred milliliters o[illegible]g 0.1% SDS onto the membrane. Cool to room temperature.

If a membrane is to be reprobed, it must not be allowed to dry out between hybridization and stripping. If it becomes dry, the probe may bind to the matrix.

2. Place membrane on a sheet of dry Whatman 3MM filter paper and blot excess liquid with a second sheet. Wrap the membrane in plastic wrap and set up an autoradiograph.

If signal is still seen after autoradiography, rewash using harsher conditions.

3. The membrane can now be rehybridized. Alternatively, it can be dried and stored for later use.

Membranes can be stored dry between Whatman 3MM paper for several months at room temperature. For long-term storage, place the membranes in a desiccator at room temperature or 4°C.

REAGENTS AND SOLUTIONS

Aqueous prehybridization/hybridization (APH) solution

5× SSC (*APPENDIX 2*)
5× Denhardt solution (*APPENDIX 2*)
1% (w/v) SDS

Add 100 µg/ml denatured salmon sperm DNA (see below) just before use

Alternatives to Denhardt solution and denatured salmon sperm DNA as blocking agents are listed in Table 2.10.5 (see discussion in critical parameters).

Denatured salmon sperm DNA

Dissolve 10 mg Sigma type III salmon sperm DNA (sodium salt) in 1 ml water. Pass vigorously through a 17-G needle 20 times to shear the DNA. Place in a boiling water bath for 10 min, then chill. Use immediately or store at −20°C in small aliquots. If stored, reheat to 100°C for 5 min and chill on ice immediately before using.

Formamide prehybridization/hybridization (FPH) solution

5× SSC (*APPENDIX 2*)
5× Denhardt solution (*APPENDIX 2*)
50% (w/v) formamide
1% (w/v) SDS

Add 100 µg/ml denatured salmon sperm DNA (see above) just before use

Alternatives to Denhardt solution and denatured salmon sperm DNA as blocking agents are listed in Table 2.10.5 (see discussion in critical parameters).

Commercial formamide is usually satisfactory for use. If the liquid has a yellow color, deionize as follows: add 5 g of mixed-bed ion-exchange resin [e.g., Bio-Rad AG 501-X8 or 501-X8(D) resins] per 100 ml formamide, stir at room temperature for 1 hr, and filter through Whatman no. 1 paper.

CAUTION: *Formamide is a teratogen. Handle with care.*

Labeling buffer

200 mM Tris·Cl, pH 7.5
30 mM $MgCl_2$
10 mM spermidine

Mild stripping solution

5 mM Tris·Cl, pH 8.0
2 mM EDTA
0.1× Denhardt solution (*APPENDIX 2*)

Moderate stripping solution

200 mM Tris·Cl, pH 7.0
0.1× SSC (*APPENDIX 2*)
0.1% (w/v) SDS

Nucleotide mix

2.5 mM ATP
2.5 mM CTP
2.5 mM GTP
20 mM Tris·Cl, pH 7.5
Store at –20°C

COMMENTARY

Background Information

Hybridization between complementary polynucleotides was implicit in the Watson-Crick model for DNA structure and was initially exploited, via renaturation kinetics, as a means of studying genome complexity. In these early applications, the two hybridizing molecules were both in solution—an approach that is still utilized in "modern" techniques such nuclease protection transcript mapping (*UNITS 4.6 & 4.7*) and oligonucleotide-directed mutagenesis (Chapter 8). The innovative idea of immobilizing one hybridizing molecule on a solid support was first proposed by Denhardt (1966) and led to methods for identification of specific sequences in genomic DNA (dot blotting; *UNIT 2.9B*) and recombinant clones (*UNITS 6.3 & 6.4*). A second dimension was subsequently introduced by Southern (1975), who showed how DNA molecules contained in an electrophoresis gel could be transferred to a membrane (*UNIT 2.9A*), enabling genetic information relating to individual restriction fragments to be obtained by hybridization analysis.

Since the pioneering work of Denhardt and Southern, advances in membrane hybridization have been technical rather than conceptual. As reviewed by Dyson (1991), the detailed protocols have become more sophisticated, largely because of advances in understanding of the factors that influence hybrid stability and hybridization rate.

Hybrid stability is expressed as the melting temperature or T_m, which is the temperature at which the probe dissociates from the target DNA. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984):

$$T_m = 81.5°C + 16.6 (\log M) + 0.41(\%GC) - 0.61 (\%form) - {}^{500}/_{L}$$

and for RNA-DNA hybrids from the equation of Casey and Davidson (1977):

$$T_m = 79.8°C + 18.5(\log M) + 0.58(\%GC) - 11.8(\%GC)^2 - 0.56(\%form) - {}^{820}/_{L}$$

where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, %form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The practical considerations that arise from these two equations are summarized Table 2.10.2A.

The second important consideration in hybridization analysis is the rate at which the hybrid is formed. Hybrid formation cannot occur until complementary regions of the two molecules become aligned, which occurs purely by chance; however, once a short nucleating region of the duplex has formed, the remaining sequences base-pair relatively rapidly. The rate at which the probe "finds" the target, which is influenced by a number of factors (Table 2.10.2B), is therefore the limiting step in hybrid formation (Britten and Davidson, 1985). However, in practical terms hybridization rate is less important than hybrid stability, as in most protocols hybridization is allowed to proceed for so long that factors influencing rate become immaterial.

Critical Parameters

To be successful, a hybridization experiment must meet two criteria:

(1) *Sensitivity.* Sufficient probe DNA must anneal to the target to produce a detectable signal after autoradiography.

(2) *Specificity.* After the last wash, the probe must be attached only to the desired target sequence (or, with heterologous probing, family of sequences).

Parameters influencing these two criteria are considered in turn, followed by other miscellaneous factors that affect hybridization.

Factors influencing sensitivity

The sensitivity of hybridization analysis is determined by how many labeled probe molecules attach to the target DNA. The greater the number of labeled probe molecules that anneal, the greater the intensity of the hybridization signal seen after autoradiography.

Probe specific activity. Of the various factors that influence sensitivity, the one that most frequently causes problems is the specific ac-

tivity of the probe. Modern labeling procedures, whether nick translation, random oligonucleotide priming (*UNIT 3.5*), or in vitro RNA synthesis (alternate protocol), routinely provide probes with a specific activity of $>10^8$ dpm/μg. This is the minimum specific activity that should be used in hybridization analysis of genomic DNA, even if the target sequences are multicopy. If the specific activity is $<10^8$ dpm/μg, hybridization signals will be weak or possibly undetectable, and no amount of adjusting the hybridization conditions will compensate. If there is a problem in obtaining a specific activity of $>10^8$ dpm/μg, it is important to troubleshoot the labeling protocol before attempting to use the probe in hybridization analysis.

If the probe is labeled to 10^8 to 10^9 dpm/μg, it will be able to detect as little as 0.5 pg of target DNA. Exactly what this means depends on the size of the genome being studied and the copy number of the target sequence. For human genomic DNA, 0.5 pg of a single-copy sequence 500 bp in length corresponds to 3.3 μg of total DNA. This is therefore the minimum amount of human DNA that should be used in a dot blot or Southern transfer if a single-copy gene is being sought.

Amount of target DNA. There is, however, a second argument that dictates that rather more than 3.3 μg of DNA should be loaded with each dot or Southern blot. During hybridization, genuine target sequences (100% homologous to the probe) and heterologous target sequences (related but not identical to the probe) compete with one another, with the homologous reactions always predominant. Ideally this competition should be maintained until the end of the incubation period so that maximum discrimination is seen between homologous and heterologous signals. This occurs only if the membrane-bound DNA is in excess, so that target sequences are continually competing for the available probe (Anderson and Young, 1985). If the probe is in excess then the homologous reaction may reach completion (i.e., all genuine target sites become filled) before the end of the incubation, leaving a period when only

Table 2.10.2 Factors Influencing Hybrid Stability and Hybridization Rate[a]

Factor	Influence
A. Hybrid stability[b]	
Ionic strength	T_m increases 16.6°C for each 10-fold increase in monovalent cations between 0.01 and 0.40 M NaCl
Base composition	AT base pairs are less stable than GC base pairs in aqueous solutions containing NaCl
Destabilizing agents	Each 1% of formamide reduces the T_m by about 0.6°C for a DNA-DNA hybrid. 6 M urea reduces the T_m by about 30°C
Mismatched base pairs	T_m is reduced by 1°C for each 1% of mismatching
Duplex length	Negligible effect with probes >500 bp
B. Hybridization rate[b]	
Temperature	Maximum rate occurs at 20-25°C below T_m for DNA-DNA hybrids, 10-15°C below T_m for DNA-RNA hybrids
Ionic strength	Optimal hybridization rate at 1.5 M Na^+
Destabilizing agents	50% formamide has no effect, but higher or lower concentrations reduce the hybridization rate
Mismatched base pairs	Each 10% of mismatching reduces the hybridization rate by a factor of two
Duplex length	Hybridization rate is directly proportional to duplex length
Viscosity	Increased viscosity increases the rate of membrane hybridization; 10% dextran sulfate increases rate by factor of ten
Probe complexity	Repetitive sequences increase the hybridization rate
Base composition	Little effect
pH	Little effect between pH 5.0 and pH 9.0

[a]This table is based on Brown (1991) with permission from BIOS Scientific Publishers.

[b]There have been relatively few studies of the factors influencing membrane hybridization. In several instances extrapolations are made from what is known about solution hybridization. This is probably reliable for hybrid stability, less so for hybridization rate.

heterologous hybridization occurring and during which discrimination between the homologous and heterologous signals becomes reduced. The problem is more significant with a double-stranded rather than a single-stranded probe, as with double-stranded probe reannealing between the two probe polynucleotides gradually reduces the effective probe concentration to such an extent that it always becomes limiting towards the end of the incubation.

In practical terms it is difficult to ensure that the membrane-bound DNA is in excess. The important factor is not just the absolute amount of DNA (which is dependent on the efficiency of immobilization and how many times the membrane has been reprobed) but also the proportion of the DNA that is composed of sequences (homologous and heterologous) able to hybridize to the probe. Rather than attempting complex calculations whose results may have factor-of-ten errors, it is advisable simply to blot as much DNA as possible: 10 µg is sufficient with most genomes. Assuming that the probe is labeled adequately and used at the correct concentration in the hybridization solution, a clear result will be obtained after autoradiography for a few hours with a simple genome (e.g., yeast DNA) or a few days with a more complex one (e.g., human DNA).

Labels other than ^{32}P. The discussion so far has assumed that the probe is labeled with ^{32}P. The lower emission energy of ^{35}S results in reduced sensitivity, and this isotope is in general unsuitable for hybridization analysis of genomic DNA. ^{35}S can be used only if the blotted DNA is exceptionally noncomplex (e.g., restricted plasmid DNA), or if the DNA is highly concentrated (e.g., colony and plaque blots; *UNIT 6.3*). Note that a membrane hybridized to a ^{35}S-labeled probe has to be dried before autoradiography, so probe stripping is not possible.

Nonradioactive probes are a more realistic option for hybridization analysis of genomic DNA and are becoming increasingly popular as the problems involved in their use are gradually ironed out. Their advantages include greater safety, the fact that large amounts of probe can be prepared in one batch and stored for years, and the rapidity of the detection protocols. Their main disadvantage is that the sensitivity of most nonradioactive detection systems is lower than that of ^{32}P autoradiography, which means that the blot and hybridization have to be carried out at maximum efficiency if a satisfactory signal is to be seen. For details on hybridization analysis with nonradioactive probes, see *UNITS 3.1b* & [illegible] and Mundy et al. (1991).

Using an inert polymer to increase sensitivity. In addition to adjusting the parameters discussed above, an improvement in sensitivity can also be achieved by adding an inert polymer such as 10% (w/v) dextran sulfate (molecular weight 500,000) or 8% (w/v) PEG 6000 to the hybridization solution. Both induce an increase in hybridization signal, 10-fold with a single-stranded probe and as much as 100-fold if the probe is double-stranded (Wahl et al., 1979; Amasino, 1986). The improvement is thought to arise from formation of interlocked meshes of probe molecules, which anneal en masse at target sites. Increased hybridization signals are a major bonus in detecting single-copy sequences in complex genomes, but this must be balanced with the fact that the polymers make the hybridization solutions very viscous and difficult to handle.

Factors influencing specificity

Ensuring specificity in homologous hybridization experiments. The hybridization incubation is carried out in a high-salt solution that promotes base-pairing between probe and target sequences. In 5× SSC, the T_m for genomic DNA with a GC content of 50% is about 96°C. Hybridization is normally carried out at 68°C, so the specificity of the experiment is not determined at this stage. Specificity is the function of post-hybridization washes, the critical parameters being the ionic strength of the final wash solution and the temperature at which this wash is carried out.

The highly stringent wash conditions described in the basic and alternate protocols should destabilize all mismatched heteroduplexes, so that hybridization signals are obtained only from sequences that are perfectly homologous to the probe. For DNA and RNA probes (as opposed to oligonucleotides), problems with lack of specificity after the highly stringent wash occur only if the hybridizing sequences are very GC-rich, resulting in a relatively high T_m. If the high-stringency wash does not remove all nonspecific hybridization, temperature can be increased by a few degrees. The equations above for calculating T_m can be used as a guide for selecting the correct temperature for the final wash, but trial and error is more reliable. Note that a membrane that has been autoradiographed can be rewashed at a higher stringency and put back to expose again, the only limitation being decay of the label and the need for a longer exposure the second time.

Table 2.10.3 High-Salt Solutions Used in Hybridization Analysis

Stock solution	Composition
20× SSC	3.0 M NaCl/0.3 M trisodium citrate
20× SSPE[a]	3.6 M NaCl/0.2 M NaH_2PO_4/0.02 M EDTA, pH 7.7
Phosphate solution[b]	1 M $NaHPO_4$, pH 7.2[c]

[a]SSC may be replaced with the same concentration of SSPE in all protocols.

[b]Prehybridize and hybridize with 0.5 M $NaHPO_4$ (pH 7.2)/1 mM EDTA/7% SDS [or 50% formamide/0.25 M $NaHPO_4$ (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS]; perform moderate-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/5% SDS; perform high-stringency wash in 40 mM $NaHPO_4$ (pH 7.2)/1 mM EDTA/1% SDS.

[c]Dissolve 134 g $Na_2HPO_4 \cdot 7H_2O$ in 1 liter water, then add 4 ml 85% H_3PO_4. The resulting solution is 1 M Na^+, pH 7.2.

Designing washes for heterologous hybridization. Calculations of T_m become more critical if heterologous probing is being attempted. If the aim is to identify sequences that are merely related, not identical, to the probe (e.g., members of a multigene family, or a similar gene in a second organism), then it is useful to have an idea of the degree of mismatching that will be tolerated by a "moderate-" or "low-" stringency wash. The best way to approach this is to first establish the lowest temperature at which only homologous hybridization occurs with a particular SSC concentration. Then assume that 1% mismatching results in a 1°C decrease in the T_m (Bonner et al., 1973) and reduce the temperature of the final wash accordingly (for example, if sequences with ≥90% similarity with the probe are being sought, decrease the final wash temperature by 10°C). If the desired degree of mismatching results in a wash temperature of <45°C, then it is best to increase the SSC concentration so that a higher temperature can be used. Doubling the SSC concentration results in a ~17°C increase in T_m, so washes at 45°C in 0.1× SSC and 62°C in 0.2× SSC are roughly equivalent. Note that in these extreme cases it may also be necessary to reduce the hybridization temperature to as low as 45°C (aqueous solution) or 32°C (formamide solution).

This approach sometimes works extremely well (as shown when the heterologous targets are eventually sequenced), but the assumption that a 1% degree of mismatching reduces the T_m of a heteroduplex by 1°C is very approximate. Base composition and mismatch distribution influence the actual change in T_m, which can be anything between 0.5° and 1.5°C per 1% mismatch (Hyman et al., 1973). Unfortunately trial and error is the only alternative to the "rational" approach described here.

Other parameters relevant to hybridization analysis

Length of prehybridization and hybridization incubations. The protocols recommend prehybridization for 3 hr with nitrocellulose and 15 min for nylon membranes. Inadequate prehybridization can lead to high backgrounds, so these times should not be reduced. They can, however, be extended without problem.

Hybridizations are usually carried out overnight. This is a rather sloppy aspect of the procedure, because time can have an important influence on the result, especially if, as described above, an excess amount of a single-stranded probe is being used. The difficulties in assigning values to the parameters needed to calculate optimum hybridization time has led to the standard "overnight" incubation, which in fact is suitable for most purposes. The exception is when hybridization is being taken to its limits, for instance in detection of single-copy sequences in human DNA, when longer hybridization times (up to 24 hr) may improve sensitivity if a single-stranded probe is being used. Note that this does not apply to double-stranded probes, as gradual reannealing results in only minimal amounts of a double-stranded probe being free to hybridize after ~8 hr of incubation.

Formamide hybridization buffers. Formamide destabilizes nucleic acid duplexes, reducing the T_m by an average of 0.6°C per 1% formamide for a DNA-DNA hybrid (Meinkoth and Wahl, 1984) and rather less for a DNA-RNA hybrid (Casey and Davidson, 1977; Kafatos et al., 1979). It can be used at 50% concentration in the hybridization solution, reducing the T_m so that the incubation can be carried out at a lower temperature than needed with an aqueous solution. Originally formamide was used with nitrocellulose membranes as a means of prolonging their lifetime, as the

lower hybridization temperature results in less removal of target DNA from the matrix. More recently formamide has found a second use in reduction of heterologous background hybridization with RNA probes. RNA-DNA hybrids are relatively strong, and heterologous duplexes remain stable even at high temperatures. The destabilizing effect of formamide is therefore utilized to maximize the discrimination between homologous and heterologous hybridization with RNA probes.

Formamide probably confers no major advantage on DNA-DNA hybridization with a nylon membrane. In fact it introduces two problems, the hazardous nature of the chemical itself, and an apparent reduction in hybridization rate. The latter point is controversial (Hutton, 1977), but for equivalent sensitivity a formamide hybridization reaction usually has to incubate for longer than an aqueous one.

Alternatives to SSC. Although SSC has been used in hybridization solutions for many years, there is nothing sacrosanct about the formulation, and other salt solutions can be employed (Table 2.10.3). There is little to choose between these alternatives. SSPE and phosphate solutions have a greater buffering power and may confer an advantage in formamide hybridization solutions. Alternatively, the buffering power of SSC can be increased by adding 0.3% (w/v) tetrasodium pyrophosphate.

Probe length. Probe length has a major influence on the rate of duplex formation in solution hybridization (Wetmur and Davidson, 1986), but the effect is less marked when the target DNA is immobilized. In membrane hybridization a more important factor is the specificity of the probe. The probe should never be too long (>1000 bp), as this increases the chance of heterologous duplexes remaining stable during a high-stringency wash. Neither should the probe contain extensive vector sequences, as these can hybridize to their own target sites, wrecking the specificity of the experiment.

Mechanics of hybridization. Traditionally hybridization has been carried out in plastic bags. This technique is messy, radiochemical spills being almost unavoidable, and can lead to detrimental contact effects if too many membranes are hybridized in a single bag. Hybridization incubators are now available from a number of companies and are recommended as a distinct advance over the plastic bag technology. Rotation of the hybridization tube results in excellent mixing, reducing hot spots caused by bubbles and dust and leading to very evenly hybridized membranes. Good reproducibility results are possible even when ten or more minigel Southerns are hybridized in a single 8.5 × 3.0–inch tube.

If bags are used, they should be of stiff plastic to prevent the sides collapsing on to the membrane, which will lead to high background. The volume of hybridization solution should be sufficient to fill the bag, and no more than two membranes should be hybridized in each bag.

Troubleshooting

Problems in blotting and hybridization reveal themselves when the autoradiograph is developed. A guide to the commonest problems and how to solve them is given in Table 2.10.4 (based on Dyson, 1991).

A particularly troublesome problem is high background signal across the entire membrane. This is due to the probe attaching to nucleic acid binding sites on the membrane surface, the same sites that bind DNA during the blotting procedure. Prehybridization/hybridization solutions contain reagents that block these sites and hence reduce background hybridization. The most popular blocking agent is Denhardt solution, which contains three polymeric compounds (Ficoll, polyvinylpyrrolidone, and BSA) that compete with nucleic acids for the membrane-binding sites. The formulations used in the basic and alternate protocols also include denatured salmon sperm DNA (any complex DNA that is nonhomologous with the target is acceptable) which also competes with the probe for the membrane sites. Blocking agents are included in the prehybridization solution to give them a head start over the probe. With a nylon membrane, the blocking agents may have to be left out of the hybridization solution, as they can interfere with the probe-target interaction. When the membranes are washed, the Denhardt solution and salmon sperm DNA are replaced with SDS, which acts as a blocking agent at concentrations ≥1%.

Other blocking agents can also be used (Table 2.10.5). With DNA blots, the main alternatives to Denhardt are heparin (Singh and Jones, 1984) and milk powder (BLOTTO; Johnson et al., 1984), although Denhardt is generally more effective, at least with nylon membranes. Note that BLOTTO contains RNases and so can be used only in DNA-DNA hybridizations. With an RNA probe, denatured salmon sperm DNA is sometimes replaced by 100 µg/ml yeast tRNA, which has the advantage that it does not need to be sheared before

Table 2.10.4 Troubleshooting Guide for DNA Blotting and Hybridization Analysis[a]

Problem	Possible cause[b]	Solution
Poor signal	Probe specific activity too low	Check labeling protocol if specific activity is $<10^8$ dpm/μg.
	Inadequate depurination	Check depurination if transfer of DNA >5 kb is poor.
	Inadequate transfer buffer	1. Check that 20× SSC has been used as the transfer solution if small DNA fragments are retained inefficiently when transferring to nitrocellulose. 2. With some brands of nylon membrane, add 2 mM Sarkosyl to the transfer buffer. 3. Try alkaline blotting to a positively charged nylon membrane.
	Not enough target DNA	Refer to text for recommendations regarding amount of target DNA to load per blot.
	Poor immobilization of DNA	See recommendations in *UNIT 2.9A* commentary.
	Transfer time too short	See recommendations in *UNIT 2.9A* commentary.
	Inefficient transfer system	Consider vacuum blotting as an alternative to capillary transfer.
	Probe concentration too low	1. Check that the correct amount of DNA has been used in the labeling reaction. 2. Check recovery of the probe after removal of unincorporated nucleotides. 3. Use 10% dextran sulfate in the hybridization solution. 4. Change to a single-stranded probe, as reannealing of a double-stranded probe reduces its effective concentration to zero after hybridization for 8 hr.
	Incomplete denaturation of probe	Denature as described in the protocols.
	Incomplete denaturation of target DNA	When dot or slot blotting, use the double denaturation methods described in *UNIT 2.9B*, or blot onto positively charged nylon.
	Blocking agents interfering with the target-probe interaction	If using a nylon membrane, leave the blocking agents out of the hybridization solution.
	Final wash was too stringent	Use a lower temperature or higher salt concentration. If necessary, estimate T_m as described in *UNIT 6.4*.
	Hybridization temperature too low with an RNA probe	Increase hybridization temperature to 65°C in the presence of formamide (see alternate protocol).
	Hybridization time too short	If using formamide with a DNA probe, increase the hybridization time to 24 hr.
	Inappropriate membrane	Check the target molecules are not too short to be retained efficiently by the membrane type (see Table 2.9.1).

continued

Table 2.10.4 Troubleshooting Guide for DNA Blotting and Hybridization Analysis, continued

Problem	Possible cause[b]	Solution
	Problems with electroblotting	Make sure no bubbles are trapped in the filter-paper stack. Soak the filter papers thoroughly in TBE before assembling the blot. Used uncharged rather than charged nylon.
Spotty background	Unincorporated nucleotides not removed from labeled probe	Follow protocols described in *UNIT 3.4*.
	Particles in the hybridization buffer	Filter the relevant solution(s).
	Agarose dried on the membrane	Rinse membrane in 2× SSC after blotting.
	Baking or UV cross-linking when membrane contains high salt	Rinse membrane in 2× SSC after blotting.
Patchy or generally high background	Insufficient blocking agents	See text for of discussion of extra/alternative blocking agents.
	Part of the membrane was allowed to dry out during hybridization or washing	Avoid by increasing the volume of solutions if necessary.
	Membranes adhered during hybridization or washing	Do not hybridize too many membranes at once (ten minigel blots for a hybridization tube, two for a bag is maximum).
	Bubbles in a hybridization bag	If using a bag, fill completely so there are no bubbles.
	Walls of hybridization bag collapsed on to membrane	Use a stiff plastic bag; increase volume of hybridization solution.
	Not enough wash solution	Increase volume of wash solution to 2 ml/10 cm^2 of membrane.
	Hybridization temperature too low with an RNA probe	Increase hybridization temperature to 65°C in the presence of formamide (see alternate protocol).
	Formamide needs to be deionized	Although commercial formamide is usually satisfactory, background may be reduced by deionizing immediately before use.
	Labeled probe molecules are too short	1. Use a ^{32}P-labeled probe as soon as possible after labeling, as radiolysis can result in fragmentation. 2. Reduce amount of DNase I used in nick translation (*UNIT 3.5*).
	Probe concentration too high	Check that the correct amount of DNA has been used in the labeling reaction.
	Inadequate prehybridization	Prehybridize for at least 3 hr with nitrocellulose or 15 min for nylon.
	Probe not denatured	Denature as described in the protocols.
	Inappropriate membrane type	If using a nonradiocative label, check that the membrane is compatible with the detection system.
	Hybridization with dextran sulfate	Dextran sulfate sometimes causes background hybridization. Place the membrane between Schleicher and Schuell no. 589 WH paper during hybridization, and increase volume of hybridization solution (including dextran sulfate) by 2.5%.

continued

Table 2.10.4 Troubleshooting Guide for DNA Blotting and Hybridization Analysis[a], continued

Problem	Possible cause[b]	Solution
	Not enough SDS in wash solutions	Check the solutions are made up correctly.
Extra bands	Final wash was not stringent enough	Use a higher temperature or lower salt concentration. If necessary, estimate T_m as described in *UNIT 6.4*.
	Probe contains nonspecific sequences (e.g., vector DNA)	Purify shortest fragment that contains the desired sequence.
	Target DNA is not completely restriction digested	Check the restriction digestion (*UNIT 3.1*).
	Formamide not used with an RNA probe	RNA-DNA hybrids are relatively strong but are destabilized if formamide is used in the hybridization solution.
Nonspecific background in one or more tracks	Probe is contaminated with genomic DNA	Check purification of probe DNA. The problem is more severe when probes are labeled by random printing. Change to nick translation.
	Insufficient blocking agents	See text for of discussion of extra/alternative blocking agents.
	Final wash did not approach the desired stringency	Use a higher temperature or lower salt concentration. If necessary, estimate T_m as described in *UNIT 6.4*.
	Probe too short	Sometimes a problem with probes labeled by random priming. Change to nick translation.
Cannot remove probe after hybridization	Membrane dried out after hybridization	Make sure the membrane is stored moist between hybridization and stripping.
Decrease in signal intensity when reprobed	Poor retention of target DNA during probe stripping	1. Check calibration of UV source if cross-linking on nylon. 2. Use a less harsh stripping method (support protocol).

[a]Based on Dyson (1991).

[b]Within each category, possible causes are listed in decreasing order of likelihood.

use. If a cDNA clone is used as the probe, or for the in vitro synthesis of an RNA probe, then blockage of sites with high affinity for poly(A)$^+$ sequences often reduces background. This is achieved by using 10 μg/ml of poly(A) DNA as the blocking agent.

Anticipated Results

Using either a nitrocellulose or nylon membrane and a probe labeled to $\geq 10^8$ dpm/μg, there should be no difficulty in detecting 10 pg of a single copy sequence in human DNA after 24 hr autoradiography.

Time Considerations

The hybridization experiment can be completed in 24 hr, the bulk of this being taken up by the overnight incubation. Prehybridization takes 3 hr for a nitrocellulose membrane or 15 min for nylon. Post-hybridization washing to high stringency can usually be completed in 1.5 hr. If a single-copy sequence in human DNA is being probed, the hybridization step may be extended to 24 hr, with a concomitant increase in the length of the experiment as a whole.

The length of time needed for autoradiography depends on the abundance of the target sequences in the blotted DNA. Adequate exposure can take anything from overnight to several days.

Table 2.10.5 Alternatives to [illegible]nhardt/Denatured Salmon Sperm DNA as Blocking Agents in DNA Hybridization[a]

Blocking agent	Composition	Storage and use
BLOTTO	5% (w/v) nonfat dried milk/0.02% (w/v) NaN_3 in H_2O	Store at 4°C; use at 4% final concentration
Heparin (porcine grade II)	50 mg/ml in 4× SSC	Store at 4°C. Use at 500 µg/ml with dextran sulfate or 50 µg/ml without
Yeast tRNA	10 mg/ml in H_2O	Store at 4°C; use at 100 µg/ml
Homopolymer DNA	1 mg/ml poly(A) or poly(C) in H_2O	Store at 4°C; use at 10 µg/ml in water; appropriate targets: poly(A) for AT-rich DNA, poly(C) for GC-rich DNA

[a]This table is based on Brown (1991) with permission from BIOS Scientific Publishers.

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Key Reference

Dyson, N.J. 1991. See above.

Provides a detailed account of factors influencing hybridization.

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